REVIEW

# Effect of trehalose on protein structure

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**Abstract: Trehalose is a ubiquitous molecule that occurs in lower and higher life forms but not in mammals. Till about 40 years ago, trehalose was visualized as a storage molecule, aiding the release of glucose for carrying out cellular functions. This perception has now changed dramatically. The role of trehalose has expanded, and this molecule has now been implicated in a variety of situations. Trehalose is synthesized as a stress-responsive factor when cells are exposed to environmental stresses like heat, cold, oxidation, desiccation, and so forth. When unicellular organisms are exposed to stress, they adapt by synthesizing huge amounts of trehalose, which helps them in retaining cellular integrity. This is thought to occur by prevention of denaturation of proteins by trehalose, which would otherwise degrade under stress. This explanation may be rational, since recently, trehalose has been shown to slow down the rate of polyglutamine-mediated protein aggregation and the resultant pathogenesis by stabilizing an aggregation-prone model protein. In recent years, trehalose has also proved useful in the cryopreservation of sperm and stem cells and in the development of a highly reliable organ preservation solution. This review aims to highlight the changing perception of the role of trehalose over the last 10 years and to propose common mechanisms that may be involved in all the myriad ways in which trehalose stabilizes protein structures. These will take into account the structure of trehalose molecule and its interactions with its environment, and the explanations will focus on the role of trehalose in preventing protein denaturation.**

**Keywords: anhydrobiosis; carbohydrates; protein structure; trehalose**

## Introduction

The use of osmolytes in the stabilization of biomolecules is an old trick of nature. These small molecules counteract the various stress conditions that an organism encounters. The mechanism by which this stabilization occurs is not fully understood; in fact, there may not be a universal rule that works for all compatible solutes. These solutes range from sugars to polyols, amino acids and their derivatives, and so forth.[1,2] Among these, the interest in trehalose has shown periodic crests and troughs with various theories being advanced to explain the astonishing properties that have, at times, been attributed to it. Although a number of articles reporting the stabilization of various biomolecules by trehalose (and in some cases, sucrose) have appeared in the recent literature, none of them has attempted to unravel the common thread that may link the various studies together. For example, studies that concentrate on the physical or chemical properties of trehalose and its solution pay little attention to finding out a correlation with biological activity.[3–11] Similarly, articles reporting gene expression profiling relevant to trehalose as a bioprotector do not make a

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**Table I.** *Physical and Chemical Properties of Trehalose*

| Parameter | Value |
|---|---|
| Chemical formula | $C_{12}H_{22}O_{11}$ (anhydrous)<br>$C_{12}H_{22}O_{11}\cdot 2H_2O$ (dihydrate) |
| Molecular weight | 342.3 g $mol^{-1}$ (anhydrous)<br>378.3 g $mol^{-1}$ (dihydrate) |
| Appearance | White, odourless powder |
| Sweetness (relative) | 45% that of sucrose |
| Polymorphs | Two crystalline forms: anhydrous ($T_\alpha$, $T_\beta$) and dihydrate ($T_h$), one well-defined amorphous form |
| Chemical nature | Nonreducing disaccharide (α-D-glucopyranosyl-α-D-glucopyranoside) |
| Relative density | 1.22 g $cm^{-3}$ (at 25°C and weight fraction = 0.5) |
| Glass transition temperature | 117°C |
| Thermostability | >99% (120°C/90 min) |
| Melting point | 203°C (anhydrous)<br>97°C (dihydrate) |
| Heat of fusion | 53.4 kJ $mol^{-1}$ (anhydrous)<br>57.8 kJ $mol^{-1}$ (dihydrate) |
| Hygroscopicity | None at <90% RH |
| Solubility in water | 68.9 g/100 g (at 20°C) |
| Freezing temperature (100 mg $mL^{-1}$ water) | −197°C |
| Optical rotation | $[\alpha]_D = +178°C$ |
| Viscosity | 8.2 cp (at 40°C and weight fraction = 0.5) |
| Chemical reactivity | Does not usually react with proteins |
| Regulatory status | GRAS (generally regarded as safe) by US FDA |

RH, relative humidity.

serious attempt to relate the protective action to physical and chemical properties of the molecule.[12–17] This review attempts to bridge this gap and to find out how data from physicists, chemists, and life scientists can be collected and collated so that an understanding of general interest to protein scientists may emerge.

Trehalose is a white, odorless powder with relative sweetness 45% that of sucrose. It is a bisacetal, nonreducing homodisaccharide in which two glucose units are linked together in a α-1,1-glycosidic linkage (α-D-glucopyranosyl-α-D-glucopyranoside; mycose, mushroom sugar). Some of the properties of trehalose are listed in Table I. Because of the inherent properties of trehalose, namely prevention of starch retrogradation and stabilization of proteins and lipids, it has proved quite useful in a number of industries including food processing, cosmetics, pharmaceutics, and so forth.[18,19] The restraining factor in the industrial use of trehalose has been the high cost of manufacturing the sugar. With the development of an enzyme-based method (from *Arthrobacter* sp. Q36) for trehalose synthesis,[20] the use is expected to increase. US Food and Drug Administration granted generally recognized as safe status to the disaccharide in 2000, which will certainly increase the scope of the areas where this disaccharide is being used.

Trehalose is present quite widely in the biological world[14,19] and performs different functions (Table II). For example, in prokaryotes, trehalose is thought to serve as a carbon source, a structural component, or as a compatible solute in halophiles and cyanobacteria.[47] In *Escherichia coli*, trehalose is synthesized in response to high osmolarity.[22] On the other hand, *Bacillus subtilis* utilizes trehalose only as a carbon source and not for osmoregulation.[21] The disaccharide is also present in many other species of bacteria, including *Streptomyces*, where trehalose is stored in the aerial hyphae and spores.[24] It forms a part of the cell wall in different species of mycobacteria, including *Mycobacterium tuberculosis* and the phylogenetically related corynaebacteria (including *Corynebacterium glutamicum*).[12,23] Free trehalose is also found in the latter during hyperosmotic stress.

In the animal kingdom, trehalose has been found in very high amounts in the adult roundworm (about 6% of the dry weight) and in the eggs of roundworm (about 9% of the dry weight).[47] In fact, tardigrades and other anhydrobiotic organisms have been proposed as model organisms for space research because of their inherent resistance to various harmful stimuli, an effect that is attributed mainly to a high level of trehalose in their systems.[48] Trehalose has not been found in higher vertebrates to date even though the enzyme required to hydrolyze trehalose (trehalase) has been reported. In humans, the enzyme is found in the brush border cells of the epithelial membrane of the small intestine and in the proximal tubules of the kidneys.[49,50] Their role is probably limited to the metabolism of any ingested trehalose and was elucidated when a patient with inherited trehalase deficiency suffered from diarrhoea following intake of mushrooms.[51] The presence of trehalase is surprising, since humans

**Table II.** *Applications of Trehalose in Various Areas of Stress Protection*

| Applications | Reference(s) |
|---|---|
| In nature | |
| Carbon/energy source | 21 |
| Osmoregulation | 22 |
| Structural component of bacterial cell wall | 12,23 |
| Dessication protectant | 24,25 |
| Cryoprotection | 13 |
| Immunogenicity (cord factor in mycobacteria) | 26 |
| Growth regulator in plants | 27,28 |
| In laboratory | |
| Treatment of xerophthalmia and xeroderma | 29,30 |
| Inhibition of osteoclast differentiation as a model for treatment of osteoporesis | 31 |
| Suppression of "senior" body odor | 20 |
| Stabilization of drug molecules against stress | 32,33 |
| Stabilization of vaccines and liposomes | 34 |
| Drought resistance in crop plants | 15 |
| Preservation of mammalian cells against dessication | 35 |
| Protection against thermal stress | 36 |
| Cryoprotection of human fibroblasts and oocytes | 37,38 |
| Stabilization of model phospholipids against leakage | 39 |
| Protection against hypoxia in mammalian cells | 40 |
| Protection against anoxia-induced protein aggregation | 41 |
| Huntington's disease mouse model | 42 |
| Cell line model of OPMD | 43 |
| Osteoporesis in mouse models | 44 |
| Aggregation of β-amyloid | 45 |
| Aggregation of prion protein | 46 |

Since nature is the source of clues, the table lists some of the areas where trehalose occurs naturally and exhibits its protective action against exposure of the organism to abiotic stress. This is followed by some examples where trehalose has been used as a "designer" protectant in different fields.

lack another hydrolase, namely cellulase, even though the corresponding substrate, cellulose, is much more widespread than trehalose.

## Physical Properties of Trehalose

The solid state and solution properties of trehalose impart to it the characteristics that are held to be responsible for the bioprotective role of trehalose. In this section, we describe some of the more widely accepted theories that may explain its extraordinary properties. Many more conjectures abound, all of them with more criticism than support, and will not be discussed here.

### *Solid state polymorphs*

One of the most important reasons why trehalose is such an important bioprotectant is due to the existence of a number of polymorphs, both in the crystalline as well as amorphous states. At least two fully crystalline forms and several more transitory intermediates between crystalline and amorphous forms are known[52–55] (see Fig. 1). The most commonly occurring crystalline form, trehalose dihydrate (depicted as $T_h$), is stable at room temperature.[55] Careful dehydration of the dihydrate under defined conditions leads to the formation of the anhydrous crystal (depicted as $T_\beta$).[56] Dehydration of the dihydrate by heating at temperatures below 85°C, at low scanning rates, results in another anhydrous form (depicted as $T_\alpha$).[57] The low rate of scanning results in a low rate of water loss, ensuring that the crystalline lattice does not degenerate into a more relaxed form. This transformation is reversible, and the anhydrous form can be hydrated back to the dihydrate form without any loss of integrity of the crystalline structure. The anhydrous crystalline form $T_\alpha$ is thought to absorb moisture, undergoing a reversible transition to the crystalline dihydrate form.[54] This reversibility, without alteration of the three-dimensional structure of the disaccharide, is important in its protective action.

### *Glass transition temperature*

Glass transition temperature ($T_g$), the temperature above which transition from viscous to fluid state occurs and the components acquire greater mobility, is the property that is invoked the most while explaining the "unusual effects" exhibited by trehalose.[25,58,59] Melts of carbohydrates can be cooled to room temperature without undergoing crystallization. This transition to the glassy state or the vitrification theory has been put forward as the most widely accepted hypothesis to explain the bioprotective action of trehalose.[58,60,61] Glasses typically have very high viscosities, with the result that any molecule that is encapsulated by a glassy matrix becomes, for all practical purposes,

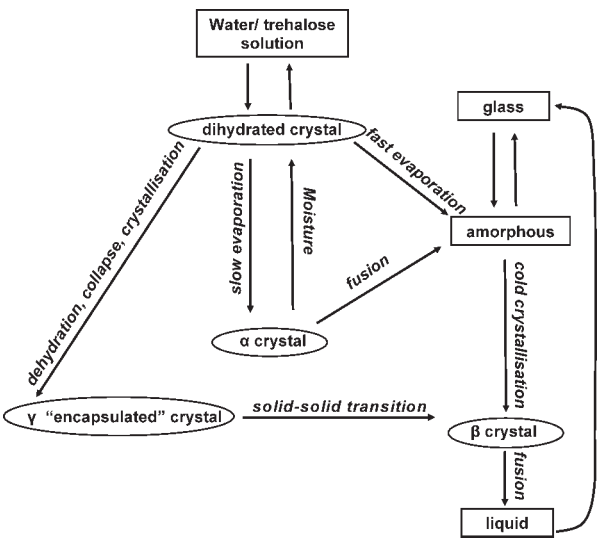


**Figure 1.** Transformation path of trehalose forms. Dihydrate trehalose $T_h$ is formed at equilibrium from the solution state and can be transformed reversibly to $T_\alpha$. In addition, depending on the temperature and rate of water loss, $T_h$ can give either an amorphous form or the $T_\gamma$ form. Reprinted from Ref. 54, with permission from Elsevier.

immobile and hence stable when exposed to stress conditions. Though controversy still reigns over the exact mechanism by which trehalose acts as a desiccation-protectant, two points of view have emerged; these need not be mutually exclusive. In the case of "immobilization theory," trehalose is viewed as a cocoon that traps the biomolecule inside a glassy matrix, like amber-encasing insects.[58,62] The movement of proteins is restricted by the sugar matrix. As discussed earlier, trehalose can transit between one crystalline form and another, without relaxing its structural integrity.[54] Trehalose has the highest glass transition temperature of all the disaccharides.[63,60] In general, addition of water to an amorphous substance increases its mobility leading to a decrease in $T_g$. Though this anticipated decrease does occur in the case of trehalose, $T_g$ is still much higher than sucrose or maltose, neither of which exhibit so many polymorphic forms.[63] This is probably the reason why trehalose is preferred over sucrose even though the latter has been postulated to play a similar role in some plants. In amorphous trehalose, local pockets of crystalline dihydrate exist, which trap residual water molecules, immobilizing them when water is scarce. This, along with the reversible transition between the dihydrate and anhydrous crystalline forms, is proposed to be the mechanism by which trehalose traps a biomolecule inside it and protects it during desiccation.[6] Partial evidence for this hypothesis has recently been provided using positron annihilation lifetime spectroscopy to study the effect of dehydration on trehalose.[6] In the glassy state, the average size of the free volume hole between trehalose molecules increases with increasing water activity, pointing to the role of water as a plasticizer by breaking hydrogen bonds between trehalose molecules. On the other hand, when water is removed from $T_\alpha$ anhydrous crystals, the channels become increasingly elongated. Under limiting water conditions, the channels are visualized as infinitely long cylindrical holes. Thus, the "anomalous" properties of trehalose are ascribed to the high stability of the glassy state and a specific interaction with water molecules in the crystalline state.[6] Data for glass transition and melting of trehalose/water systems are highly disparate and make comparison of results from different groups difficult.[63] It needs to be emphasized that the $T_g$ of trehalose is not unusual but is at the highest end of all disaccharides and in the same league as tetrasaccharides.[54] $T_g$ of a trehalose solution is higher than that of a sucrose solution, at all water activities. Thus, a sample incubated along with trehalose and stored at 37°C is still around 80°C below its $T_g$ (assuming the $T_g$ of trehalose to be ~117°C, a widely accepted value),[63] whereas the same sample, in the presence of sucrose, will be only 28°C below its $T_g$ (which is 65°C). Because of the higher $T_g$ of trehalose as compared to sucrose, a sample incubated with sucrose is more likely to absorb moisture, resulting in a decrease in its $T_g$ to the storage temperature (37°C in the above case), which will cause the sample to degrade. Since the $T_g$ of trehalose is much higher than sucrose, even if a sample containing trehalose were to absorb moisture and its $T_g$ were to decrease, it will still not reach a stage where the $T_g$ goes below the storage temperature of 37°C. That is how trehalose proves to be a better antidote to stress exposure. A trehalose containing sample, on absorbing water, produces more of the dihydrate crystalline form. Other disaccharides like α-melibiose monohydrate, α-lactose monohydrate, and so forth are also capable of transition to the crystalline hydrate but do not show similar bioprotective effects during desiccation and/or other stress conditions.[61] Trehalose has also been implicated in the protection of organisms against cold-induced stress. Restoration of *otsA*/*otsB* genes from an *E. coli* deletion mutant for these two genes leads to an expected increase in trehalose production and also to an increase in the viability of the mutant at 4°C.[64] It is hypothesized that trehalose prevents the inactivation and aggregation of proteins at lower temperature and stabilizes the cell membrane by delaying the onset of phase shift from liquid crystal to gel state.[64] Trehalose is also thought to increase the cytoplasmic viscosity, thus decreasing the possibility of formation of intracellular ice crystals which are often fatal.[65]

### *Solution properties*

Trehalose has been classified as a kosmotrope or water-structure maker, that is the interaction between trehalose/water is much stronger than water/water interaction and may be involved in its bioprotective action.[66] Raman scattering experiments reveal the disruption of the tetrahedral network of water molecules on addition of trehalose.[9] This "destructuring" of the water network by trehalose and ordering the water molecules around itself (as a kosmotrope) does not allow the formation of ice and makes trehalose one of the best cryoprotectants known. Maltose and sucrose follow trehalose in this behavior. Above a specific concentration (30%), sugar molecules force their hydrogen-bonding imprint on the water network, and trehalose turns out to be the most successful in this as is clear from the decrease in the "open" band intensity [ν(OH)].[9] The decrease in the band intensity for the open conformation (O—H vibrational mode in tetrabonded water molecule) with increasing concentration of disaccharides reiterates the destructuring effect of the disaccharides on the tetrahedral hydrogen bonded network of water. Thirty percent appears to be the threshold value of concentration beyond which the disaccharides, especially trehalose, form their own hydrogen-bonded network with water and the disruption caused by the disaccharide becomes more apparent. Thus, trehalose is capable of disturbing the bonding arrangement of water more than maltose or sucrose, decreasing the freezing temperature of

biological solutions to a lower value than the other two disaccharides. This is consistent with the earlier observation that disaccharides hamper crystallization of proteins by decreasing the amount of freezable water.[67] Molecular dynamic (MD) simulations have confirmed that the addition of disaccharides to water leads to a drastic reorganization of the hydrogen bonded network of the latter and a significant increase in the fraction of population forming hydrogen bonds.[5,9] This effect is again much more pronounced for trehalose than sucrose or maltose and is attributed to the higher hydration number for trehalose. The pattern of water distribution around the glycosidic oxygen is found to be different for all the disaccharides and is also different from other oxygens in disaccharides.[5] The characteristic first solvation peak at 2.8 Å, which is well defined in all the other cases, is found to be missing for the glycosidic oxygens of the three (1,1)-linked disaccharides (including trehalose) under study.[5] Instead, this peak shifts to 3.5 Å for trehalose, with lowered peak density. Thus, the glycosidic oxygen is not easily hydrated as compared to the rest of the oxygens, and this effect is most pronounced in the case of trehalose. Also, the radial distribution function around the glycosidic oxygen in trehalose shows restricted distribution of water molecules when compared with other oxygens and other disaccharides. The hydration number of the glycosidic oxygen is lower than all other oxygens and is particularly low in the case of trehalose (0.028 when compared with an average value of 0.153 for the glycosidic oxygen in other disaccharides).[5] Thus, water molecules are nonuniformly distributed around oxygens of disaccharides, and this distribution plays an important role in the behavior of trehalose. MD simulations reveal that the dynamic motion about the glycosidic linkage is much slower in trehalose than in all other disaccharides in aqueous solutions. (The lifetimes of internal motions about the glycosidic linkage in case of trehalose, isotrehalose, and neotrehalose are 4.74, 3.13, and 3.16 ns, respectively. This value is 2.89 ns in case of maltose).[5] Since the lability of the glycosidic linkage is probably the most important parameter in determining the conformation, these results, coupled with results indicating ordered distribution of water around the glycosidic oxygen of trehalose, explain how trehalose is able to maintain its conformation even in the aqueous milieu. The preferential exclusion theory has been cited in situations where water is in excess.[68–70] According to this, trehalose, or any other sugar molecule, does not interact directly with the biological macromolecule. Water is said to be excluded from the solvation layer of the biomolecule and is ordered around trehalose. With increase in the concentration of trehalose in bulk water, there is a competition between trehalose and the biomolecule for the available water. This competition causes water molecules to be destructured around biomolecules and "structured" around trehalose, making it a kosmotrope or water structure-maker. It is possible that depending on the structure of the protein/biomolecule in question, trehalose will be able to manipulate the water structure around itself, such that the protein/biomolecule is stabilized. Though the distribution of water molecules around trehalose is not uniform, they are oriented around trehalose in such a way that an ordered structure, with hydrogen bonds in all directions, is formed.

Indirect evidence for the manipulation of surrounding water layer by trehalose has also come from NMR and ultrasonic studies which reveal that trehalose has a very high affinity for water.[71,72] NMR relaxation time measurements show that trehalose has the highest hydration ability among all the saccharides studied. Although trehalose lacks internal hydrogen bonds, at very high concentrations, the two glucose rings fold over, forming intermolecular hydrogen bonds.[71,72,73] Ultrasonic studies support this hypothesis since the compressibility of trehalose solutions strongly depends on concentration.[72] As water is added, unfolding occurs, making hydrogen bonding sites available for water molecules. Thus trehalose offers its protective action by reducing the activity of water. Trehalose orders the water network, at least up to the third hydration shell, reducing the amount of freezable water. According to the water replacement theory, which is an alternate hypothesis presented to explain the unique properties of trehalose, the disaccharide is thought to substitute water around the biomolecule, maintaining its three-dimensional structure by providing sites with hydrogen-bonding species.[74–77] This substitution also restricts the mobility of the biological macromolecule. Direct experimental evidence is available for this. When freeze-dried porcine pancreatic lipase is redissolved in water, the enzyme becomes flexible and active (and also susceptible to thermal denaturation). When suspended in any organic solvent, on the other hand, the enzyme shows very low activity. If freeze-drying is carried out in the presence of trehalose or a two-component system such as polyethylene glycol–trehalose, the same enzyme shows very high activity in organic medium as well.[78] This is because the water molecules are replaced with trehalose molecules that provide hydrogen-bonding network, maintaining the three-dimensional structure of the active enzyme. How much of the interaction is a direct one between trehalose and the biomolecule remains doubtful. The mechanism of stabilization by trehalose or any other cosolute during freeze-drying is infinitely more complex than simple cryoprotection.[34,79] Many cosolutes which act as efficient cryoprotectants fail to protect the protein from drying/desiccation-induced stress. The only exceptions are disaccharides.[16,80,81] FT-IR studies carried out with trehalose and proteins have confirmed that hydrogen bonding does occur between the sugar and the protein and that this is a requirement for the preservation of

labile proteins during desiccation. In the case of lysozyme, the position of the amide I band shifts from 1652 $cm^{-1}$ for the fully hydrated enzyme to 1659 $cm^{-1}$ when it is lyophilized in the absence of any excipients. In the presence of trehalose, the band regains the original position it occupied for the enzyme in solution.[80] Similarly, the shift in the amide II band from 1543 $cm^{-1}$ (for the fully hydrated enzyme) to 1530 $cm^{-1}$ (for the enzyme lyophilized in the absence of any excipients) is reversed when lyophilization is carried out in the presence of trehalose. At higher concentrations of trehalose, sublimation results in crystallization of the disaccharide, and hydrogen bonding is no longer possible. Under these conditions, the spectrum of lysozyme resembles that of the enzyme freeze-dried in the absence of trehalose.[80] Thus, hydrogen bonding is a prerequisite for stabilization of proteins by trehalose during freeze-drying. The stabilization to stress experienced by a protein during lyophilization is complicated by the fact that the stabilizer excipient should have both cryoprotective as well as lyoprotective properties. It is here that trehalose emerges the winner. A monosaccharide like glucose might be an efficient lyoprotectant but it provides no protection against freezing.[82] Trehalose, on the contrary, offers both cryo- as well as lyoprotection during freeze-drying. It stabilizes the partially unfolded state of the protein (rather than stabilizing the native structure).[83] Trehalose is thought to replace the water shell around proteins/membranes and to preserve the three-dimensional structure of the biomolecule.[75] There is a huge body of evidence in support of this viewpoint as well, especially when proteins are freeze-dried for preservation. FT-IR and other studies have pointed to the gradual replacement of water by sugars, mainly trehalose, but also polymers like polyethylene glycol, and so forth.[84–87] In many cases where comparisons have been carried out, trehalose has come out at the top, not only among disaccharides but in comparison with other excipients as well.[86,87] Simultaneous use of trehalose and ionic salts has been shown to have a synergistic effect on the freeze-drying stability of hen egg white lysozyme.[88] This stabilization results from the interaction of trehalose with the protein, as confirmed by changes in the CO stretching vibration, resulting from the destructuring of the surrounding water molecules. This study also serves as a reminder that, during freeze-drying, the mixture does not remain homogeneous and a concentration gradient of components of the lyophilization mixture is set up.[88] Proposing any hypothesis needs to take into account this often-ignored fact. Recent studies on structural relaxation times with human growth hormone have shown only a weak correlation between the secondary structure and aggregation propensity of the protein.[89] This case is one of the few exceptions where addition of sucrose results in a stable, though more mobile, formulation than trehalose. There may be other reasons for this observation. Since trehalose is a nonreducing sugar, there is no free aldehyde group available to undergo a Maillard-type reaction with amino groups of proteins. Additionally, the α-1,1-glycosidic bond in trehalose is much less susceptible to hydrolysis than the α,β-1,2 glycosidic bond of the other nonreducing sugar, sucrose. In fact, under suboptimal conditions, the behavior of sucrose resembles that of glucose, and the rate of condensation reaction is ~2000 times faster than with trehalose.[90] This could be a major reason why trehalose is preferred over sucrose when a protein is lyophilized or when an organism faces desiccation, both scenarios where water availability is low.

The data presented earlier make it quite clear that trehalose, by virtue of its structure, has acquired some extraordinary properties. However, one question that begs an answer is as follows: are solution properties enough? It is difficult to see how the characteristics of trehalose in aqueous solution will help explain the stabilization effect observed when it is used as an excipient during freeze-drying. In this case, the vitrification properties of trehalose assume importance. Though the changes occurring during the initial replacement of water by trehalose are relevant, it is perhaps judicious to consider the changes that trehalose itself may face in low water conditions before looking at it as a stabilizer for biomolecules under low water conditions. In other words, the mechanism by which trehalose offers stabilization to a biomolecule in water-excess medium is probably different from the mechanism by which it does so in a low-water situation.

## Biopreservative Action of Trehalose

A few areas where trehalose is active have been listed in Table II. This section deals specifically with cases where interaction of trehalose with proteins is involved. A number of polyhydroxyl compounds, including sugars, are used by organisms to overcome stress conditions like heat, cold, desiccation, and so forth. Trehalose, and to some extent sucrose, constitute a major category of these molecules. Their importance lies in the manner in which they can stabilize proteins as well as lipid bilayer. In the case of proteins, the most commonly invoked hypothesis to explain the transition from native to denatured state, in terms of thermodynamic parameters, is Tanford's transfer model.[91] The basis of this model is that energetics of osmolytes on protein stability is an additive term of the free-energy contributions of polar and nonpolar groups exposed on the native and denatured states of a protein. As shown,[92,93] when the solubility of a protein in 1*M* osmolyte solution is greater than in water, the solubility ratio (of the protein in water and 1*M* osmolyte) is less than 1, the apparent transfer free energy is negative, and the interaction between the protein and the osmolyte is energetically favorable. If this ratio is more than 1, the interaction between the protein and the osmolyte becomes unfavorable. As

pointed out by the authors, unfavorable interaction of trehalose with the peptide backbone of the partially formed intermediate causes preferential exclusion of the osmolyte from the protein–water interface, thus increasing its free energy.[92] This is complemented by a favorable interaction of trehalose with the peptide backbone of the native protein, which increases the thermodynamic barrier between the native and the partially folded forms. In fact, it has been shown recently that the hydration of the peptide backbone in the presence of an osmolyte depends on the type of osmolyte and the size of the side chains.[94] The effect of this exclusion on the equilibrium constant of unfolding is mathematically expressed by the Wyman linkage relationship.[95] According to this, the change in the equilibrium constant due to a change in the osmolyte concentration is expressed as the difference in the number of osmolyte molecules bound by the folded and unfolded protein molecules. As stated earlier, the unfavorable interaction of the peptide backbone with the osmolyte has been identified as the thermodynamic force responsible for the increase in chemical potential of the system.[92] The unfolded state has more solvent-exposed surface area than the folded state, resulting in preferential exclusion and increase in chemical potential. The unfolded state is thus destabilized with respect to the folded state. This relationship, though widely accepted, does not take into account the effect of the osmolyte on the protein structure. Studies on the stabilization of the ribosomal protein S6 by trehalose have shown that trehalose does not alter the two-state character of the equilibrium unfolding of the protein.[96] The stabilization induced by trehalose is nonspecific and is not localized to any particular secondary structure motif. This also agrees well with the change in transfer of free energy on unfolding and the stabilization inferred from thermal transitions.[96] In addition, trehalose favors the collapse of the unfolded state coupled with an increase in the rate of folding and a decrease in the rate of unfolding of the protein.[96] In case of unfolding of cutinase by heat, trehalose increases the melting temperature of the enzyme by delaying thermal unfolding of an intermediate.[97] Trehalose compensates for the altered entropy of the process by increasing the contribution of enthalpy to the process, making unfolding a thermodynamically unfavorable process.

On exposure to heat, yeast produces trehalose, along with heat shock proteins, on a large scale.[98] It has now been shown in yeast that trehalose stabilizes partially folded proteins during heat shock and is rapidly hydrolyzed on activation of the partially folded form by molecular chaperones.[99] Trehalose offers protection to yeast glucose-6-phosphate dehydrogenase and phosphoglucoisomerase against thermoinactivation.[100] Induction of thermotolerance by trehalose is also inferred from the fact that the level of trehalose is correlated with thermotolerance.[100] Surprisingly, trehalose has also been found to inhibit aggregation of denatured proteins (e.g., bovine glutamic dehydrogenase) following heat shock.[100] Whether trehalose aids the function of the chaperone or vice versa is not clear. In some cases, trehalose is thought to influence chaperone activity of heat shock proteins. There is evidence that heat shock leads to increased synthesis of components of the trehalose-6-phosphate synthase complex in *Saccharomyces cerevisiae*.[101] Yeast mutants defective in genes for trehalose anabolism display much lower survival rates than the native species.[36] Adding confusion to this controversy are reports which indicate that the synthesis of heat shock proteins is neither necessary nor sufficient for the induction of thermotolerance in yeast.[102,103] On the other hand, *E. coli* mutants with no *otsA* or *otsB* do display reduced thermotolerance.[104] More conclusive proof can be derived from the study of the effect of trehalose on the thermoinactivation of enzymes. In one study, trehalose has been found to protect yeast pyrophosphatase against exposure at 50°C much better than other sugars like sucrose, fructose, or glucose.[105] This is attributed to the lower concentration of water in trehalose solutions. A 1.5*M* trehalose solution contains 62.5% water by volume; at a similar molar concentration, sucrose and maltose solutions contain 87% and 86% water by volume, respectively. This enhanced hydrated volume of trehalose results in increased viscosity. If the volumetric concentrations of water in sucrose and maltose solutions are equalized to that in a 1.5*M* trehalose solution, the respective concentrations required turn out to be 4.3*M* and 4*M*. Since these disaccharides are insoluble at such high molar concentrations, a 4.4*M* glycerol solution is prepared, which contains 62.5% water. At this concentration, the levels of thermoprotection afforded to pyrophosphatase by both trehalose and glycerol are identical,[105] proving that it is the hydrated volume and not the molar concentration per se that determines the amount of stabilization offered. Fluorescence emission studies have confirmed that both trehalose and glycerol prevent unfolding of the enzyme when used at the same volumetric concentration. When used at the same molar concentration as trehalose, both sucrose and maltose fail to prevent the unfolding of the enzyme at 50°C to any significant extent.[105] It is possible that trehalose and other sugars solubilize in the bulk water and are excluded from the solvation layer of the protein (preferential exclusion theory). This leads to a decrease in the solvation layer around the protein, restricting its mobility, and stabilizing it against stress conditions. As the hydrated radius of a trehalose molecule is ~2.5 times higher than that of sucrose or maltose, the size-exclusion effects become more pronounced and the stabilization effect is high.[105]

One of the most exciting areas where trehalose has shown some promise is in therapeutics. Trehalose has already proved its usefulness as a protectant

against oxidative stress. As stated earlier, trehalose accumulation can be induced in *S. cerevisiae* following exposure to gentle stress conditions such as mild heat shock[98] or the presence of a proteasome inhibitor.[106] These cells show increased tolerance when exposed to a free radical generating system. Mutants unable to synthesize trehalose show exceedingly low viability when exposed to oxidative stress. This effect can be reversed if trehalose is made available to the mutants.[106] The reaction of oxygen radicals with proteins gives rise to free carbonyl groups in the latter. It is hypothesized that exposure to $H_2O_2$ causes aggregation of proteins and that the presence of trehalose overcomes this damage, presumably by acting as a free radical scavenger. Trehalose-deficient mutants of *Candida albicans* are extremely sensitive to exposure to $H_2O_2$ while the same condition induces trehalose accumulation in wild-type cells, with improved life spans.[107] In mammalian cells, $O_2$ deprivation or hypoxia for 5–10 min results in irreversible tissue damage.[40] *Drosophila* however can survive exposure to complete $N_2$ atmosphere for up to 4 h without any damage. In this case, overexpression of *TPS1* gene leads to accumulation of trehalose with concomitant increase in tolerance to $O_2$ deprivation.[41] Trehalose is also believed to reduce anoxia-induced protein aggregation *in vitro*.[41] Transfection of *Drosophila TPS1* gene into HEK-293 cells has given rise to populations that show decreased trehalose metabolism when grown in glucose-deficient media. These transfected cells show increased trehalose accumulation when exposed to hypoxic stress and show much higher survival rates as compared to control cells. The amount of aggregated as well as ubiquitinated proteins in the transfected cells is much less when exposed to $O_2$-depleted atmosphere.[41] Thus stabilization of proteins by trehalose is probably responsible for survival of lower organisms when exposed to hypoxic stress.

Expansion of glutamine units in many proteins results in their aggregation leading to neurodegenerative diseases. One of these is the Huntington's disease where expansion of CAG units occurs in the first exon of the gene coding for huntingtin protein.[108] Although the premise that protein aggregation is a precursor of the disease is debatable, cell culture studies have shown that amelioration of aggregation does lead to relieving the complications in the cellular machinery which ultimately cause cell death. A mutant myoglobin has been engineered, which has an expanded polyglutamine stretch (35 residues) and is a good model for polyglutamine diseases.[42] A library of small molecules has been screened in vitro for their ability to inhibit protein aggregation caused by the presence of expanded polyglutamine units. The compounds chosen are such that they are nontoxic and suitable for oral administration. Disaccharides emerge as successful candidates with, perhaps not surprisingly, trehalose surfacing as the one which causes the most significant reduction in aggregation in vitro.[42] Other disaccharides such as sucrose, maltose, and melibiose do cause a reduction in aggregation but the effect is marginal as compared to trehalose which exhibits a dose-dependent behavior. The same results can be reproduced in mouse neuroblastoma cell lines expressing a truncated N-terminal huntingtin containing 150 glutamine residues, fused to an enhanced green fluorescent protein (EGFP).[42] Interestingly, the trehalose metabolite glucose does not show any significant effect, confirming that it is trehalose itself and not its ability to act as a source of glucose which is responsible for the alleviation of symptoms. Increasing the concentration of trehalose does not affect the expression of polyglutamine-containing truncated huntingtin. Overexpression of *E. coli otsA* and *otsB* results in the accumulation of trehalose in the above cell line and inhibition of intracellular aggregation. Oral administration of trehalose (at 0.05*M*) to R6/2 transgenic mice (a mouse model of Huntington's disease) reduces dilatation of lateral ventricles.[42] The average brain weight of the transgenic mice also increases. More importantly, immunohistochemical staining (for ubiquitin-positive protein aggregates) confirms the decrease in intranuclear aggregation in motor cortex, striatum, and liver upon administration of trehalose.[42] As mentioned earlier, trehalose is mostly impermeable to plasma membrane. However, it can easily be loaded into mammalian cells using fluid-phase endocytosis and pinocytosis[109] by simply being in contact with the cells, as in this case, via oral administration of this molecule. The stabilization of an engineered myoglobin containing 35 glutamine residues upon administration of trehalose is of the same order as that of an engineered myoglobin containing a nonpathogenic stretch of glutamine residues. Thus, the stabilization may have resulted from the interaction of the polyglutamine stretch with trehalose rather than the truncated N-terminal huntingtin, which does not fold easily even in the presence of trehalose. This is so since trehalose has no effect on the stability of wild-type, nonengineered myoglobin. It is also important to recollect that trehalose is rapidly hydrolyzed once the stabilized partially folded protein is taken over by chaperones for correct and complete folding.[99] Trehalose, in fact, has been shown to inhibit protein folding. Thus, in all probability, trehalose interacts with the expanded polyglutamine stretch and stabilizes it early before aggregation can set in.

Another codon reiteration disease is oculopharyngeal muscular dystrophy (OPMD), which is caused by the abnormal expansion of the polyalanine stretch in the poly(A) binding nuclear protein 1 (PABPN1).[110] The expanded GCG repeat units in the *PABPN1* gene express a protein that aggregates as tubular filaments within the nuclei of skeletal muscle fibres. These aggregates are presumed to be toxic and are crucial in OPMD pathology. Trehalose has been shown to

decrease aggregation and toxicity of mutants in a COS7 cell line expressing EGFP tagged-PABPN1.[43] Transgenic A17-1 mice (mouse models of OPMD) show a decrease in aggregation (as seen by immunolabeling for PABPN1-positive aggregates in the nuclei of bicep section of treated mice) accompanied by attenuation of muscle weakness upon oral administration of trehalose. Addition of cyclohexamide (an inhibitor of protein synthesis) and lactacystin (an inhibitor of trehalose-induced clearance of mutant PABPN1) confirms that trehalose promotes the clearance of mutant PABPN1 via proteasomes.[43] No comparison with other disaccharides has been made in this study. Perhaps not surprisingly, trehalose has also been found to inhibit aggregation of β-amyloid (Aβ) to form amyloid plaques, the pathological hallmark of Alzheimer's disease, in a dose-dependent manner.[45] Interestingly, however, this inhibition follows different patterns in case of Aβ-40 and Aβ-42. While in the former, inhibition of aggregation is seen at all concentrations of trehalose studied, the effect is seen only at the highest concentration in case of Aβ-42. Also, trehalose is able to reverse the cytotoxicity caused by Aβ-40, but not by Aβ-42. This can be correlated with the retention of toxic oligomeric structures by Aβ-42 even in the presence of trehalose. Because of the presence of two more relatively nonpolar amino acid residues, Aβ-42 is thought to be more hydrophobic and hence more prone to aggregation than Aβ-40. Interaction between hydrophobic patches results in the loss of ordering of surrounding water molecules, decreasing the entropy and thus free energy of the process. Addition of trehalose cannot overcome this thermodynamic barrier and hence trehalose is unable to disassemble the toxic, oligomeric assemblies of Aβ-42.[45] There has been a recent report of trehalose reducing the size of $PrP^{Sc}$ (a protease-resistant form of the prion protein $PrP^{C}$) aggregates in prion-infected ScN2A cells and protecting these cells against oxidative stress.[46] More research is necessary however before trehalose can be prescribed as a therapeutic molecule in this case, since breaking up of infectious prion aggregates may result in formation of smaller particles that can act as nuclei for further aggregation.

Trehalose has also been found useful in relieving the symptoms of osteoporesis.[44] Symptoms of estrogen deficiency following ovariectomy in mice, namely decrease in bone weight and loss of calcium and phosphorus levels in the femur, are reversed upon oral administration of trehalose, which has no effect on the weight of uterus.[44] Thus, trehalose does not act in the same manner as estrogen but rather by the inhibition of osteoclast differentiation leading to reduced femoral bone loss.

Trehalose has also been found to act as a stabilizer to improve the shelf-life of therapeutic proteins. Work with model proteins has shown that trehalose is able to abrogate the moisture-induced aggregation of bovine serum albumin, by interfering with the formation of intermolecular disulphide bonds.[33] Electrophoresis studies with recombinant human serum albumin in the solid state have shown the formation of a completely amorphous state in the presence of trehalose and sucrose and stabilization of the protein during storage over a 4-month period at 35°C.[111] Similar kind of stabilization effects have been shown by another sugar, sorbitol, in the stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation.[32] Trehalose has also demonstrated favorable properties in the stabilization of recombinant botulinum serotype A vaccine during lyophilization[112] and IgG1 in spray-dried formulations.[113]

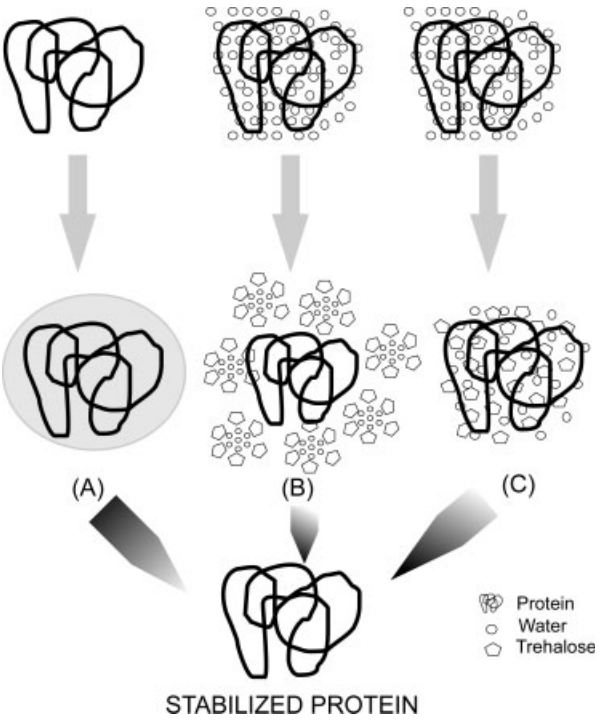


**Figure 2.** Various theories to explain the "exceptional" properties of trehalose. (**A**) Vitrification theory assumes that trehalose forms a glassy matrix that acts as a cocoon and presumably physically shields the protein or indeed cells from abiotic stresses. (**B**) Preferential exclusion theory, on the other hand, proposes that there is no direct interaction between trehalose and protein (or biomolecule). Instead, as can be seen, addition of trehalose to bulk water sequesters water molecules away from the protein, decreasing its hydrated radius and increasing its compactness and consequently stability. (**C**) Water replacement theory talks of substitution of water molecules by trehalose-forming hydrogen bonds, maintaining the three-dimensional structure and stabilizing biomolecules.

## Conclusions

The various explanations used to elucidate the bioprotective role of trehalose have been covered in sufficient detail in the previous sections. Three main rationales have emerged (see Fig. 2). None of these, on its own,

is sufficient to explain the bioprotection offered by trehalose. The situation "on the ground" is probably an amalgamation of these theories, which need not be mutually exclusive. It needs to be reiterated that none of the physical or chemical properties of trehalose discussed earlier is sufficient in itself to explain the bioprotective action that trehalose offers across the spectrum of stress conditions that an organism faces. As summarized later, these properties are neither "unusual" nor "exceptional." They lie at one end of the range (either highest or lowest) and in some cases, even in between, for disaccharides.

- Nonreducing sugar
- Relatively inert glycosidic linkage
- Absence of internal hydrogen bonds
- Existence of a number of polymorphs
- Transition between polymorphs without affecting crystallinity
- High glass transition temperature
- High fragility
- High hydrophilicity and hydration number
- Large hydrated volume
- Kosmotropy or water structure "maker"
- Restricted distribution of water molecules
- Flexibility of glucose rings to expand and contract
- Ability to increase cytoplasmic viscosity: decrease in intracellular ice formation
- Depression of melting temperature of lipids
- Stabilization of partially folded proteins

There may be individual molecules that have exceptional properties, but taken together, trehalose, with the synergistic action of all these factors, seems to offer the maximum bioprotection. It seems unlikely that there is a hitherto undiscovered parameter which will provide a solution to the "trehalose question." So, is trehalose the elixir of life? As far as countering stress conditions by organism is concerned, probably yes. Does it work because it has magical properties? No. Trehalose works simply because it possesses the best parameters that nature can provide, none of which, on its own, will at best, be capable of lending more than a helping hand. As the critical analysis mentioned earlier shows, what works is probably a cumulative effect of all the theories that have been put forward; the relative contribution of each parameter is adjusted according to the stress factor that is encountered.

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